Toxicology Letters 233 (2015) 125–137

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Influence of *in utero* di-*n*-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats

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HIGHLIGHTS

- Developmental exposure to DHP and DCHP affects testicular development in rats.
- DHP and DCHP decreased AGD and T levels of male fetuses.
- Testicular AR production disrupted after DHP and DCHP.
- The low dose effects and potential effects on AMH and possibly Sertoli cells.

ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 23 January 2015
Accepted 27 January 2015
Available online 28 January 2015

Keywords:
Di-*n*-hexyl phthalate
Dicyclohexyl phthalate
Endocrine disrupters
Aantiandrogen
Testicular development
In utero

ABSTRACT

This study investigated the effects of di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) on male reproductive development *in utero*. Pregnant rats were exposed to DHP and DCHP at doses of 0 (vehicle), 20, 100 and 500 mg/kg/day, by gavage, on gestational days (GD) 6–19. A significant decrease in the anogenital distance (AGD) of male fetuses was observed at all doses of DHP and DCHP. The AGD/cube root of body weight ratio in male fetuses was also significantly reduced compared to control group. The litters with resorption, percentage of resorptions and inhibin B levels increased in treatment groups. Moreover, testosterone and MIS/AMH levels in all treatment groups decreased. Although FSH and inhibin B levels of male pups exposed to DHP and DCHP increased, FSH/inhibin B ratio decreased in treatment groups. Reduced testosterone production in response to DHP and DCHP exposure appeared to be related to changes in testosterone metabolism, as shown by decreased 3β-HSD immunoexpression. The percentages of large Leydig clusters increased after exposure to DHP and DCHP *in utero*. Histopathological examination of the testis on GD20 revealed changes at all doses. Relative integrated immunodensities of 3β-HSD, MIS/AMH, PCNA and AR decreased after DHP and DCHP exposures. Altered fetal Sertoli cell development and function may be caused by disrupted PMC function revealed by reduced AR production in these cells in treatment groups.

1. Introduction

Phthalates are diester derivatives of phthalic acid used primarily as plasticizers in commercial products, such as plastic food wraps, children's toys, blood transfusion and dialysis bags, and catheters, to make the plastic products more flexible (Bhattacharya et al., 2005; Zhang et al., 2004). However, phthalates are not covalently bound to the plastic material and released into the environment. Phthalates are frequently detected in outdoor and indoor air (Saito et al., 2001). The possibility of these compounds entering into biologic systems has caused great concern among the public about their reproductive and developmental toxicity (Ema and Miyawaki, 2002).

Abbreviations: 3β-HSD, 3-beta-hydroxysteroid dehydrogenase; AR, androgen receptor; BBP, butylbenzyl phthalate; DBP, dibutyl phthalate; DCHP, dicyclohexyl phthalate; DEHP, di-(2-ethylhexyl) phthalate; DHP, di-*n*-hexyl phthalate; ER, estrogen receptor; FSH, follicle-stimulating hormone; GD, gestational day; LH, luteinizing hormone; MIS/AMH, Mullerian inhibiting substance/Antimullarian hormone; PCNA, proliferating cell nuclear antigen; PMC, peritubular myoid cells; PND, postnatal day; T, testosterone.

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http://dx.doi.org/10.1016/j.toxlet.2015.01.015
0378-4274/

Dicyclohexyl phthalate (DCHP) is a common plasticizer ingredient for production of nitrocellulose, ethyl cellulose, vinyl acetate, polyvinyl chloride, and resins (HSDB, 2008). Di-*n*-hexyl phthalate (DHP) is often found as a minor component (less than 1%) of C6-C10-phthalate mixtures; it may also be an isomer in mixtures of diisohexyl phthalates (DIHP) at levels of 25% or lower (Kavlock et al., 2002). Recent studies have showed that exposure to some phthalates including DHP and DCHP result in serious and irreversible changes in the development of reproductive tract of the male rat (Foster et al., 2001; Sharpe, 2001). Various study groups proposed that phthalates disrupt and alter the development of male reproductive system by a decrease in androgen production (Foster, 2006; Heger et al., 2012; Howdeshell et al., 2008a,b; Johnson et al., 2012; Mitchell et al., 2012; Saillenfait et al., 2013). Saillenfait et al. (2009b) exposed pregnant Sprague-Dawley rats to DHP or DCHP at doses of 0 (olive oil), 250, 500 and 750 mg/kg/day, by gavage, on gestational days (GD) 6–20. They found that DHP caused dose related developmental toxic effects, including marked embryo mortality at 750 mg/kg/day, and significant decreases in fetal weight at 500 and 750 mg/kg/day. They also observed a significant and dose-related decrease in the anogenital distance of male fetuses induced by DHP and DCHP. They concluded that DHP showed clear embryolethality and teratogenicity, whereas DCHP. DHP was without affect on fetal weight or mortality in that study. Another study by Saillenfait et al. (2009a) showed that DHP exposure on GD 12–21 to reduced AGD in male rat offsprings on PND1 at 125 mg/kg/day and above, and areola/nipple retention before weaning and at adulthood at 250 and 500/mg/kg/day. They also observed hypospadias, underdeveloped testis, and undescended testis in young adult male rats at 125 mg/kg/day and higher doses.

In our previous studies, we showed that *in utero* DHP and DCHP exposures affected the development of male reproductive tract at prepubertal, pubertal and adult stages of life (Aydogan Ahbab and Barlas, 2013) and exerted genotoxic effects to testicular cells of rats at all stages of development, even at adulthood (Ahbab et al., 2014). The aim of this study is to investigate the effects of di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) on the development of testis and AGD *in utero*. Pregnant Wistar rats were exposed to DHP and DCHP at doses of 0, 20, 100 and 500 mg/kg/day, by gavage, on gestational days (GD) 6–19, and the effects of these phthalates on morphology, hormonal, histopathologic changes at GD 20 were investigated.

2. Materials and methods

2.1. Chemicals

Di-*n*-hexyl phthalate (CAS no. 84-75-3; 97% purity) and dicyclohexyl phthalate (CAS no. 84-61-7; 99% purity) were supplied from Alfa Aesar Co., Inc., and Aldrich Chemical Co., and dissolved in corn oil (vehicle).

2.2. Animals and treatments

Pregnant time-mated female Wistar albino rats (two-month-old and 200–220 g weight) were considered as on gestational day 0 (GD 0 was the day when sperm detected in the vaginal lavage) were purchased from the Experimental Animals Production Center, Hacettepe University in Ankara, Turkey. All rats were housed in polycarbonate cages with stainless steel covers, and in a room maintained 12 h light/dark cycle with a temperature of $22 \pm 2\,^{\circ}C$ and a relative humidity of 50 ± 5, and given standard rat diet (Korkutelim Feed Factory, Afyon, Turkey) and tap water *ad libitum*. The pregnant rats were distributed on a random basis into control (vehicle) and treatment groups ($N = 10$) and housed individually.

The pregnant rats were administered DHP and DCHP in corn oil by gavage at dose of 0 (vehicle), 20, 100 and 500 mg/kg/day from GD 6 to 19. The solutions were prepared fresh daily according to dams' weights. The dosing volume was 0.25 ml in all groups. The rats in the vehicle control group received corn oil in equal amounts as in experimental groups. Maternal body weight, food consumption and clinical signs of toxicity of animals were recorded daily. At necropsy on GD20 in the morning, the dams euthanized by cervical dislocation and the litters with resorptions, percentage resorption [(number of resorbing embryos/total number of embryos) × 100], length (from head to end of the tail) and weight of male pups were orecorded. Anogenital distance (AGD) was measured on male pups at necropsy using digital calipers. All experimental procedures and animal use were as approved by the Approval of Ethics Committee of Hacettepe University.

2.3. Hormone analysis

At the end of the study on GD 20, fetal blood was collected by removing fetuses rapidly from the uterus followed by decapitation. Trunk blood was collected in tubes that contained heparin. Plasma was separated after centrifugation at 3000 rpm for 15 min and stored at $-80\,^{\circ}C$ until hormone analysis. Male fetal plasma in each litter was pooled, stored at $-80\,^{\circ}C$ until assayed for hormone analysis.

T (the sensitivity of the assay is 20 pg/ml) and FSH (the sensitivity of the assay is 0.5 ng/ml) (Endocrine Tech, Newark, CA), inhibin B (minimum limit of detection is 7 pg/ml) and MIS/AMH (minimum limit of detection is 0.006 ng/ml) (Diagnostic System Laboratories, Webster, TX, USA) were measured by using commercially available EIA kits according to the manufacturer's instructions. The intra- and inter-assay coefficients of variations were less than 9.1%.

2.4. Histopathological analysis

Testes samples of male pups were removed and fixed in Bouin's solution for 3 h. The tissues were embedded in paraffin, cut at 4 μm thickness, stained with Harris hematoxylin and eosin, and then examined under light microscope for histopathological evaluation and photographed using Olympus BX51 microscope with imagine program Pixera Pro 150ES. One testis from each litter was evaluated for incidence of histopathologic lesions.

2.5. Immunohistochemical staining of 3β-HSD, MIS/AMH, AR and PCNA

Sections from testes tissue samples were cut at 4 μm and processed for immunohistochemical examination by the avidin-biotin-peroxidase method by using Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). For immunohistochemistry staining, primary antibodies were used in the following dilution: 3β-HSD, 1:50; and MIS/AMH, 1:50. Briefly, the sections were deparaffinized and rehydrated in PBS for 10 min. Antigen retrieval was performed by heating slides in citrate buffer (pH 6) in water bath for 2 h at $98\,^{\circ}C$ and then cooled for 20 min. Then the slides were washed twice in phosphate buffer for 20 min. After that, endogenous peroxide activity was blocked with 30-min incubation in methanol containing 1% hydrogen peroxide for 15 min and washed twice in phosphate buffer solution (pH 7.2) for 5 min each. Tissue sections were incubated for 20 min with buffer containing 5% normal serum for protein blockage. Primary antibodies for anti-MIS/AMH (1:50 dilution; Santa Cruz, sc-6886) and anti-3β-HSD (1:50 dilution; Santa Cruz, sc-30820) were used. Primary antibodies were diluted in 5% normal serum and then the sections were incubated with primary antibodies overnight at $+4\,^{\circ}C$.